รายงานวิจัยฉบับสมบูรณ์

โครงการ

การโคลน แสดงออก และการศึกษา phylogenetic ของตัวตอบรับ Toll ของกุ้งกุลาดำ

โดย

ผู้ช่วยศาสตราจารย์ ดร.วันชัย อัศวลาภสกุล

เสร็จสิ้นโครงการเมื่อ พฤษภาคม 2554

สัญญาเลขที่ MRG5180160

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ชื่อหัวหน้าโครงการวิจัยผู้รับทุน

ผู้ช่วยศาสตราจารย์ ดร.วันชัย อัศวลาภสกุล

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ชื่อนักวิจัยที่ปรึกษา

ศาสตราจารย์เกียรติคุณสกล พันธุ์ยิ้ม

ชื่อสถาบันของนักวิจัยที่ปรึกษา

สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนงานวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานคณะกรรมการอุดมศึกษา (สกอ.) และสำนักงานกองทุนสนับสนุนงานวิจัย (สกว.) ที่ให้การสนับสนุนโครงการวิจัยนี้เสร็จสมบูรณ์

ผู้วิจัยขอขอบคุณ ศาสตราจารย์ ดร.อัญชลี ทัศนาขจร ภาควิชาชีวเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ให้คำแนะนำในการโคลนยีน Toll และความช่วยเหลือทางด้านชีวโมเลกุล และ คุณวัลลภ ชินนิรันดร์วงศ์ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ที่ให้ความช่วยเหลือด้านการเลี้ยงกุ้ง และงานทางชีวโมเลกุล

ผู้วิจัยขอขอบคุณสถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ที่ได้ให้การสนับสนุนอุปกรณ์และเครื่องมือวิทยาศาสตร์ และสถานที่ในการทำวิจัย

ผู้วิจัยขอขอบคุณภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ส่งเสริมให้บุคลากรมีความร่วมมือระหว่างมหาวิทยาลัย

สุดท้ายนี้ผู้วิจัยขอขอบคุณนักวิจัยที่ปรึกษา ศาสตราจารย์เกียรติคุณสกล พันธุ์ยิ้ม สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

ผู้ช่วยศาสตราจารย์ ดร.วันชัย อัศวลาภสกุล

สารบัญ

บทคัดย่อภาษาไทย

รหัสโครงการ : MRG5180160

ชื่อโครงการ : การโคลน แสดงออก และการศึกษา phylogenetic ของตัวตอบรับ Toll ของกุ้งกุลาดำ

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บทคัดย่อ

กุ้งกุลาดำเป็นสัตว์น้ำเศรษฐกิจของหลายๆ ประเทศ รวมถึงประเทศไทย ซึ่งระบบภูมิคุ้มกันของกุ้งมีความคล้ายคลึงกับสัตว์ที่ไม่มีกระดูกสันหลังชนิดอื่น ๆ กล่าวคือเป็นระบบภูมิคุ้มกันที่มีมาแต่กำเนิด ตัวรับ Toll หรือ Toll-like มีบทบาทสำคัญในการที่จะจดจำโมเลกุล Spätzle ที่ใช้ในการกระตุ้นระบบภูมิคุ้มกัน ในการศึกษาครั้งนี้ผู้วิจัยรายงานยีนตัวรับ Toll ของกุ้งกุลาดำ ซึ่งมีขนาด 4,144 นิวคลีโอไทด์ ประกอบด้วยส่วน 5'-UTR ขนาด 366 นิวคลีโอไทด์ และ 3'-UTR ขนาด 985 นิวคลีโอไทด์ ที่มีลำดับนิวคลีโอไทด์ AATAAA ที่เป็น polyadenylation signal และมี poly A-tail ยาว 27 นิวคลีโอไทด์ ยีนนี้แปลเป็นโปรตีน 931 กรดอะมิโน โปรตีนตัวรับ Toll ของกุ้งกุลาดำ เป็นเมมเบรนโปรตีนชนิดที่ 1 และมีสมบัติของตัวรับ Toll-like กล่าวคือ มีส่วน signal peptide, มี leucine-rich repeats domain อยู่ในส่วนที่อยู่ภายนอกเซลล์, มีส่วนเมมเบรนโปรตีน และมีส่วน TIR ที่อยู่ภายในเซลล์ โปรตีนตัวรับ Toll แสดงออกในเนื้อเยื่อต่าง ๆ ได้แก่ เหงือก เซลล์เม็ดเลือด หัวใจ ตับ ต่อมน้ำเหลือง กล้ามเนื้อ เซลล์ประสาท ขาว่ายน้ำ กระเพาะ อัณฑะ และรังไข่ เมื่อนำลำดับกรดอะมิโนของตัวรับ Toll มาศึกษา phylogenetic พบว่า มีความคล้ายคลึงกับตัวรับ Toll ในกุ้งจีนมากที่สุด อย่างไรก็ดี กลไกการทำงานของตัวรับ Toll ยังต้องมีการศึกษาต่อไป เพื่อให้เข้าใจการทำงานในการป้องกันการติดเชื้อของกุ้งกุลาดำ ซึ่งเป็นสัตว์น้ำเศรษฐกิจที่สำคัญ

คำหลัก : กุ้งกุลาดำ, ตัวรับโทลล์, การโคลน, แสดงออก, Phylogenetic

บทคัดย่อภาษาอังกฤษ

Project Code : MRG5180160

Project Title : Cloning, Express and Phylogenetic study of a Toll receptor from black tiger shrimp

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Project Period : 15 May 2008 – 30 May 2511

Abstract

The black tiger shrimp (*Penaeus monodon*) is an economically important aquatic organism in many parts of the world, including Thailand. Shrimp immunity is similar to that in other invertebrate organisms, and consists of an innate immunity. Toll or Toll-like receptors (TLRs) play an essential role in recognizing the cleaved form of the cytokine Spätzle which is processed by a series of proteolytic cascades activated by secreted recognition molecules. In this study, I report the first isolation of a full-length Toll receptor from *P. monodon*. The cloned full-length sequence of the PmToll cDNA consists of 4,144 nucleotides containing a 5'-UTR of 366 nucleotides, a 3'-terminal UTR of 985 nucleotides with a classical polyadenylation signal sequence AATAAA and a poly A-tail of 27 nucleotides, and an open reading frame coding for 931 amino acids. The deduced amino acid sequence of PmToll is a typical type I membrane domain protein, characteristic of TLRs' functional domains. It includes a putative signal peptide, an extracellular domain consisting of leucine-rich repeats (LRRs) flanked by cysteine-rich motifs, a single-pass transmembrane portion and a cytoplasmic TIR domain. The expression of PmToll was investigated in several tissues including gill, haemocyte, heart, hepatopancreas, lymphoid organs, muscle, nerve, pleopod, stomach, testis and ovary, expression was detected in all tissues. The phylogenetic relationship between the deduced amino acid of PmToll and other arthropod Tolls and the analysis suggests that PmToll is closely related to other shrimp Tolls especially FcToll. However, further studies elucidating the mechanism of action of Tolls will be of benefit in understanding the mechanism of bacterial pathogenesis of this economically important aquatic species.

Keywords : Black teger shrimp, Toll Receptor, Cloning, Express, Phylogenetic

หน้าสรุปโครงการ (Executive Summary)

ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่

ชื่อโครงการ

(ภาษาไทย) การโคลน แสดงออก และการศึกษา phylogenetic ของตัวตอบรับ Toll ของกุ้งกุลาดำ

(ภาษาอังกฤษ) Cloning, express and phylogenetic study of a Toll receptor from black tiger shrimp

1. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Thailand is the largest frozen shrimp producer and exporter in the world, generating approximately 2 billion USD annually exports value in 2003. Despite its high commercial impact, shrimp farming industry in Thailand has encountered several problems such as the outbreak of several viral diseases. Recently, RNA interference (RNAi) is a sequence-specific gene silencing mechanism in eukaryotes, which is believed to function as a defense against viruses and transposons, while removing abundant but aberrant nonfunctional messenger RNAs. Since RNAi discovery, RNAi has been developed into a widely used technique for generating genetic knock-outs and for studying gene function by reverse genetics. Additionally, inhibition of virus replication by means of induced RNAi has now been reported for numerous viruses including yellow head virus and white spot syndrome virus. The current data on RNAi-mediated inhibition of virus replication and discuss the possibilities for the development of RNAi-based antiviral therapeutics. Hence it is possible that the mechanism of RNAi could lead to a new route to protect the farm shrimps from viruses by inhibiting viral replication.

How to turn on RNAi? Usually, RNAi will be activated by any dsRNA. The interaction between dsRNA and a host protein in the cell surface can activate both specific and non-specific immune response to defense any pathogens that attack host. In case of non-specific immune response, some transcripts or some proteins that are involved in innate immune response will be activated to invading pathogens. Whereas specific immune response involves RNAi mechanism in which dsRNA will be transferred into the cell using dsRNA-binding protein before entering into RNAi pathway. Many previous studies show the RNAi mechanism has been found and applied to study in gene function and to inhibit viral replication in penaeid shrimp. In higher eukaryote, Toll-like receptors (TLRs) play a fundamental role in the recognition of bacteria fungi and viruses. TLRs harbor leucine-rich repeats (LRRs) in the extracellular portion, and a Toll/IL1 receptor (TIR) domain in the cytoplasmic portion. The TIR domain of TLRs shows high similarity with the cytolasmic region of the IL-1 receptor family. Recent accumulating evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. Thus, TIR domain-containing adaptors provide specificity of TLR signaling. The discovery of a series of TIR-containing adapters revealed that there are differences in the signal transduction pathways of individual TLRs, which might induce different effector responses that are specific to each TLR, as well as redundant

responses that are conserved in all TLRs. One of the effector's functions is to produce IFN-β, which is mediated by a TRIF-dependent pathway in TLR3 and TLR4 signaling, thus implying roles of TLRs for the detection of virus infection and the induction of appropriate anti-viral responses. Moreover, Toll-like receptor 3 has been also identified as a receptor for any double-stranded RNA. Hence, it is possible that the Toll-like receptor involves in the binding of dsRNA activating the mechanism of RNAi that lead to a new route to control any transcripts or proteins that involve in signaling and/or immune response. This project is aimed at identification of a Toll-like receptor protein that activates RNAi mechanism (dsRNA injection in shrimp) and will, therefore, lead to understanding in dsRNA-host interaction.

2. วัตถุประสงค์

Molecular cloning, tissue distribution and phylogenetic study of Toll receptor from *Penaeus monodon*

3. ระเบียบวิธีวิจัย

3.1 Cloning of Toll-like receptor from penaeid shrimp

3.1.1 Cloning of cDNA Toll receptor from penaeid shrimp and phylogenetic study

Total RNA from lymphoid organ of penaeid shrimp was isolated using standard RNA extraction method. The mRNA transcripts were converted into cDNA by reverse transcriptase using Oligo-dT link with adaptor. The cDNA was used as the template for PCR amplification by using degenerated primers designed from the conserved domain (IL1-like domain) and adaptor primer that containing restriction site. The desired PCR products were cloned into pGEM-T Easy plasmid. After screening by rapid-size screening method, the correct recombinant clones were subjected for DNA sequencing. The plasmid harboring the corrected sequence was used as template to design other primer for amplifying the full-length cDNA of TLR. The full-length cDNA was used to study in Phylogenetic tree.

3.2 Tissue distribution of Toll receptor from penaeid shrimp

To investigate the tissue distribution of PmToll, total RNA was extracted from gill, haemocyte, heart, hepatopancreas, lymphoid organ, muscle, nerve, pleopod, stomach, testis and ovary and one microgram of RNA from each tissue was used to produce first-strand cDNA with an oligo dT primer. The first-strand cDNA was used directly as a template in subsequent multiplex-PCR to amplify the PmToll gene.

4. แผนการดำเนินงานตลอดโครงการ

ปีที่ 1 ในช่วง 6 เดือนแรก : โคลน cDNA Toll receptor บางส่วน

ปีที่ 1 ในช่วง 6 เดือนหลัง : โคลน cDNA-full length ของ Toll receptor (TLR)

ปีที่ 2 ในช่วง 9 เดือนแรก : โคลน cDNA-full length ของ Toll receptor (TLR) และ ศึกษาการแสดงออกของ TLR

ปีที่ 2 ในช่วง 3 เดือนหลัง : รวบรวมข้อมูลทั้งหมด และทำการเขียน manuscript เพื่อตีพิมพ์ลงในวารสารนานาชาติที่มี impact factor

5. งบประมาณรวม 480,000 บาท โดยแบ่งเป็นหมวดต่าง ๆ ดังนี้

รายละเอียด	ปีที่ 1	ปีที่ 2	รวม 2 ปี
5.1 หมวดค่าตอบแทน	120,000	120,000	240,000
5.2 หมวดค่าวัสดุและสารเคมี	50,000	90,000	140,000
5.3 หมวดค่าใช้สอยและอื่นๆ	20,000	30,000	50,000
5.4 ค่าครุภัณฑ์	50,000	-	50,000
รวมเป็นเงิน	240,000	240,000	480,000

5.1 หมวดค่าตอบแทน เป็นจำนวนเงิน 10,000 บาท ต่อ เดือน

5.2 หมวดค่าวัสดุและสารเคมี เช่น ค่ากุ้ง, อาหารเลี้ยงกุ้ง, อุปกรณ์สำหรับเลี้ยงกุ้ง, ค่าไพร์เมอร์, ค่าบริการอ่านลำดับเบส เป็นต้น

5.3 หมวดค่าใช้สอย เช่น, อุปกรณ์สำหรับเตรียมรายงาน หรือ manuscript, reprint request, ค่าไปรษณีย์ เป็นต้น

5.4 หมวดค่าครุภัณฑ์ ได้แก่ ตู้แช่เย็น 4-8 องศาเซลเซียส และตู้แช่ -20 องศาเซลเซียส

เนื้อหางานวิจัย

Introduction

Thailand is the largest frozen shrimp producer and exporter in the world. However, the outbreak of several disease pathogens especially viral disease is a major problem in shrimp farming industry. Three penaeid viruses have been identified as causing very high mortality rates, namely yellow head virus (YHV) white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) (Assavalapsakul 2007).

Innate immunity is common to all metazoans and serves as a first-line defence. Its hallmarks are the recognition of microorganisms by germline-encoded, non-rearranging receptors, and rapid effector mechanisms that involve phagocytosis, activation of proteolytic cascades and synthesis of potent antimicrobial peptides (Hoffmann 2003). The innate immune system is of crucial importance in host defense against pathogens of invertebrates. The non-self-recognizing immune response cascade is triggered by receptors that recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, peptidoglycans and mannans, through pattern recognition receptors (PRRs). Upon recognition these receptors activate signal transduction pathways leading to the translocation of transcription factors to the nucleus and eventually control the expression of immune response genes. Depending on the pathway used the response will either be the activation of haemocytes (cellular response) or the production of antimicrobial peptides (humoral response) (Arts et al., 2007). Recent studies have revealed that insects and mammals conserve a signaling pathway of the innate immune system through cell-surface receptors called Tolls and Toll-like receptors (TLRs) (Inamori et al., 2004).

TLRs harbor leucine-rich repeats (LRRs) in the extracellular portion, and a Toll/IL1 receptor (TIR) domain in the cytoplasmic portion (Figure 1) (Kawai et al., 2005). The TIR domain of TLRs shows high similarity with the cytolasmic region of the IL-1 receptor family (Takeda et al 2004b). Recent accumulating evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 is essential for the induction of inflammatory cytokines triggered by all TLRs. TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathway. Thus, TIR domain-containing adaptors provide specificity of TLR signaling. (Takeda et al., 2004a). The discovery of a series of TIR-containing adapters revealed that there are differences in the signal transduction pathways of individual TLRs, which might induce different effector responses that are specific to each TLR, as well as redundant responses that are conserved in all TLRs. One of the effector's functions is to produce IFN-β, which is mediated by a TRIF-dependent pathway in TLR3 and TLR4 signaling, thus implying roles of TLRs for the detection of virus infection and the induction of appropriate anti-viral responses (Figure 2).

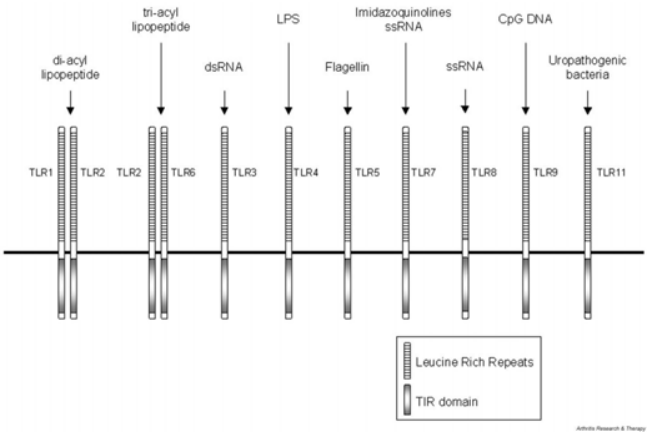


Figure 1. Structure and ligands for Toll-like receptors (TLRs). dsRNA, double-stranded RNA; LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor (Kawai et al., 2005)

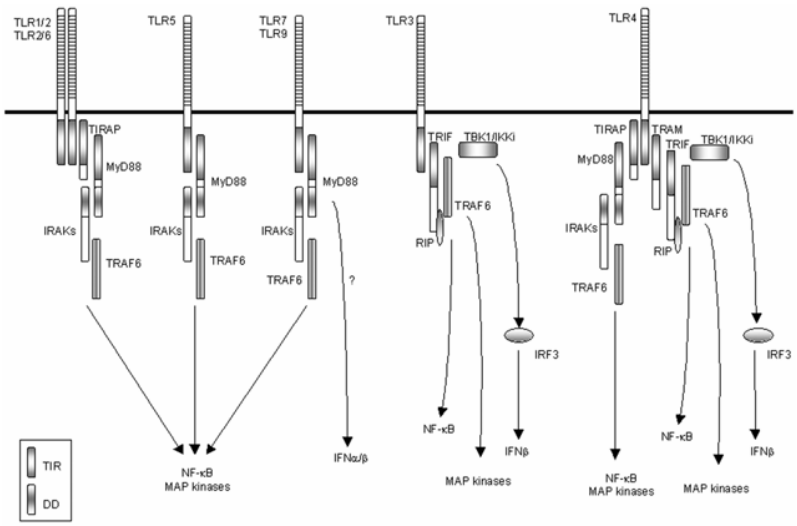


Figure 2. Schematic representation of Toll-like receptor (TLR) signaling pathways. All TLRs except for TLR3 are thought to share the MyD88-dependent pathway that activates NF-κB and mitogen-activated protein (MAP) kinases, leading to the induction of inflammatory cytokine genes. Interleukin-1 receptor-associated kinases (IRAKs) and TRAF6 are located downstream of MyD88. TIRAP is involved in the MyD88-dependent pathway downstream of TLR2 and TLR4. TRIF is utilized in the TLR3-mediated and TLR4-mediated activation of interferon regulatory factor (IRF) 3 and the subsequent induction of IRF3-dependent gene expression such as interferon-β (IFN-β). TRAM is specifically involved in the activation of IRF3 in TLR4 signaling. The complex of TBK1/IκB kinase-i (IKK-i) is responsible for the activation of IRF3 downstream of TRIF in TLR3 and TLR4 signaling. TRAF6 is also involved in the TRIF-dependent activation of NF-κB and MAP kinases. Receptor-interacting protein (RIP) mediates TRIF-dependent NF-κB activation. DD, death domain (Kawai et al., 2005).

Expression of human TLR3 in the double-stranded RNA (dsRNA)-non-responsive cell line 293 confers enhanced activation of NF-κB in response to dsRNA. In addition, TLR3-deficient mice are impaired in their response to dsRNA (Alexopoulou et al., 2001). Additionally, TLR3 contributes directly to the immune response of respiratory epithelial cells to influenza A virus and dsRNA (Guillot et al., 2005). dsRNA is produced by most viruses during their replication and induces the synthesis of type I interferons (IFN-a/b), which exert anti-viral and immunostimulatory activities. Thus, TLR3 is implicated in the recognition of dsRNA and viruses (Takeda et al., 2005)

Since its discovery, RNAi has been developed into a widely used technique for generating genetic knock-down and for studying gene function by reverse genetics. Additionally, inhibition of virus replication by means of induced RNAi has now been reported for numerous viruses, including several important shrimp pathogens such as yellow head virus (Tirasophon et al., 2005, 2007, Yodmuang et al., 2006) and white spot syndrome virus (Kim et al., 2007, Robalino et al., 2005, 2007, Westenberg et al., 2005, Xu et al., 2007). The current data on RNAi-mediated inhibition of virus replication has opened up the possibilities for the development of RNAi-based antiviral therapeutics. RNAi mechanism involves in many functional proteins that up- or down- regulated after activated-RNAi mechanism, it is possible that the mechanism of RNAi could lead to control any transcripts or proteins that involve in signaling and/or immune response. However, dsRNA-host interaction (in shrimp) still does not have reported that including in any receptor was not identified for dsRNA binding and no expression profiles were studied in shrimp after activated-RNAi mechanism.

Hence, it is possible that the mechanism of RNAi could lead to a new route to control any transcripts or proteins that involve in signaling and/or immune response. Then, this project is to identify a Toll-like receptor protein that activated RNAi mechanism (dsRNA injection in shrimp). Therefore, this study is lead to understanding in dsRNA-host interaction.

Materials and Methods

Shrimp Culture

Healthy juvenile *P. monodon* (8-10 g in weight) were purchased from a commercial farm in the domestic area and reared in a sea water tank system with a salinity of 10 parts per thousand (ppt) at 25-28 ^{o}C for 7 days.

RNA extraction

Total RNA was extracted from various tissues using TRIzol-Reagent (Invitrogen, USA) following the manufacture's protocol. The RNA concentration and its quality were determined (A_{260}) and monitored (A_{260}/A_{280} ratio > 1.8) by using NanoDrop 1000 spectrophotometer.

cDNA Cloning of PmToll

To synthesize the first-strand cDNAs, five micrograms of total RNA was subjected to reverse transcription using the M-MLV Reverse Transcription System (Fermentas, USA) according to the supplied procedure with PRT primer (Table 1). PCR was performed using the cDNA prepared as above which amplifies the initial sequence using the Toll RDW and PM1 primers (Table 1). Having isolated this partial PmToll sequence, the entire sequence was obtained using 5′-RACE-PCR with the gene-specific primers shown in Table 1. All PCR reactions were performed using 1X *Taq* Buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 2 mM each dNTPs, 2.5 U of *Taq* polymerase (Fermentas), 0.2 μM of each primer and 2 μl of template cDNA. The PCR conditions comprised 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50-55 °C for 30 s and 72 °C for 1.5 min, followed by an extension of 72 °C for 10 min. The products were cloned into the pGEM-T Easy vector and transformed into *E. coli* DH5α. Recombinant plasmids were extracted using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced commercially using the BigDye® Terminator v3.1 cycle sequencing kit (1st BASE sequencing unit, Malaysia).

Table 1. Primers used to amplify the full-length PmToll sequence of *P. monodon*

Name	Sequence (5′-->3′)
3′ RACE primers	
PmT RDW-F	TGCCTTCACTACCGCGACTGG
PM1 primer	CCGGAATTCAAGCTTCTAGAGGATCCTT
PRT primer	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTTT
5′ RACE primers	
PmT Pm-F-1	AGTGTACCTGAAGACCTCTT
PmT GSP1-R	AGCCTGGGAGTGAGCTGC
PmT GSP2-R	GAGTTCTTCCAAGCTCCTGAGATC
PmT GSP3-R	GCCTATTTGTGATGTCACTC
PM1 primer	CCGGAATTCAAGCTTCTAGAGGATCCTT
PRC primer	CGGAATTCAAGCTTCTAGAGGATCCTTGGGGGGGGGGGGGGGG
Tissue distribution of shrimp beta actin and PmToll	
PmActin F	GACTCGTACGTGGGCGACGAGG
PmActin R	AGCAGCGGTGGTCATCTCCTGCTC
PmToll F	GTCCAATCAGTTGGAGCTGC
PmToll R	GAAATCGAGCGTCTTCACATGC

Sequence Analysis and phylogenetic tree

Nucleotide sequence and deduced amino acid sequence comparisons were carried out using the BLAST algorithm at NCBI GenBank database. Sequence alignments were performed using AlignX (Vector NTI). The signal peptide was predicted using the SignalP 3.0 program (Bendtsen et al., 2004). Potential N-linked glycosylation sites were predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/). The simple modular architecture research tool (SMART) (Letunic et al., 2009) was used to analyze the deduced amino acid sequence. Phylogenetic and molecular evolutionary analyses of the predicted amino acid sequences of different Tolls were conducted using the neighbor-joining method and were drawn using *MEGA* version 4 (Tamura et al., 2007). The nucleotide sequence and deduced amino acid sequence of PmToll were submitted to GenBank (GenBank ID: GU014556 and ADK55066)

Tissue distribution of PmToll gene

To investigate the tissue distribution of PmToll, total RNA was extracted from gill, haemocyte, heart, hepatopancreas, lymphoid organ, muscle, nerve, pleopod, stomach, testis and ovary and one microgram of RNA from each tissue was used to produce first-strand cDNA with an oligo dT primer. The first-strand cDNA was used directly as a template in subsequent multiplex-PCR to amplify the PmToll gene and shrimp beta actin (internal control) by using the PmToll F and PmToll R primers and actin F and actin R primers, respectively (Table 1). The temperature profile for PCR conditions was as follows; 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. After 30 cycles, the reaction was held at 72 °C for another 10 min.

Results and discussion

In this study, we report the first isolation of a full-length Toll receptor from *P. monodon* (Figure 3). The cloned full-length sequence of the PmToll cDNA consists of 4,144 nucleotides containing a 5'-UTR of 366 nucleotides, a 3'-terminal UTR of 985 nucleotides with a classical polyadenylation signal sequence (AATAAA) and a poly A-tail of 27 nucleotides, and an open reading frame coding for 931 amino acids. The deduced amino acid sequence of PmToll is a typical type I membrane domain protein, characteristic of TLRs' functional domains. It includes a putative signal peptide (residues 1-19), an extracellular domain (residues 133-706) consisting of leucine-rich repeats (LRRs) flanked by cysteine-rich motifs, a single-pass transmembrane portion (residues 713-735) and a cytoplasmic TIR domain (residues 766-904). Twelve potential N-linked glycosylation sites, which were predicted by NetNGlyc 1.0 are located in the ectodomain. Finally, many structural features are conserved in the regions that flank the LRRs, including all of 18 cysteine residues in the LRR-CT and LRR-NT regions, and the sequence NPXXC(N/D)C in the two LRR-CT regions of PmToll.

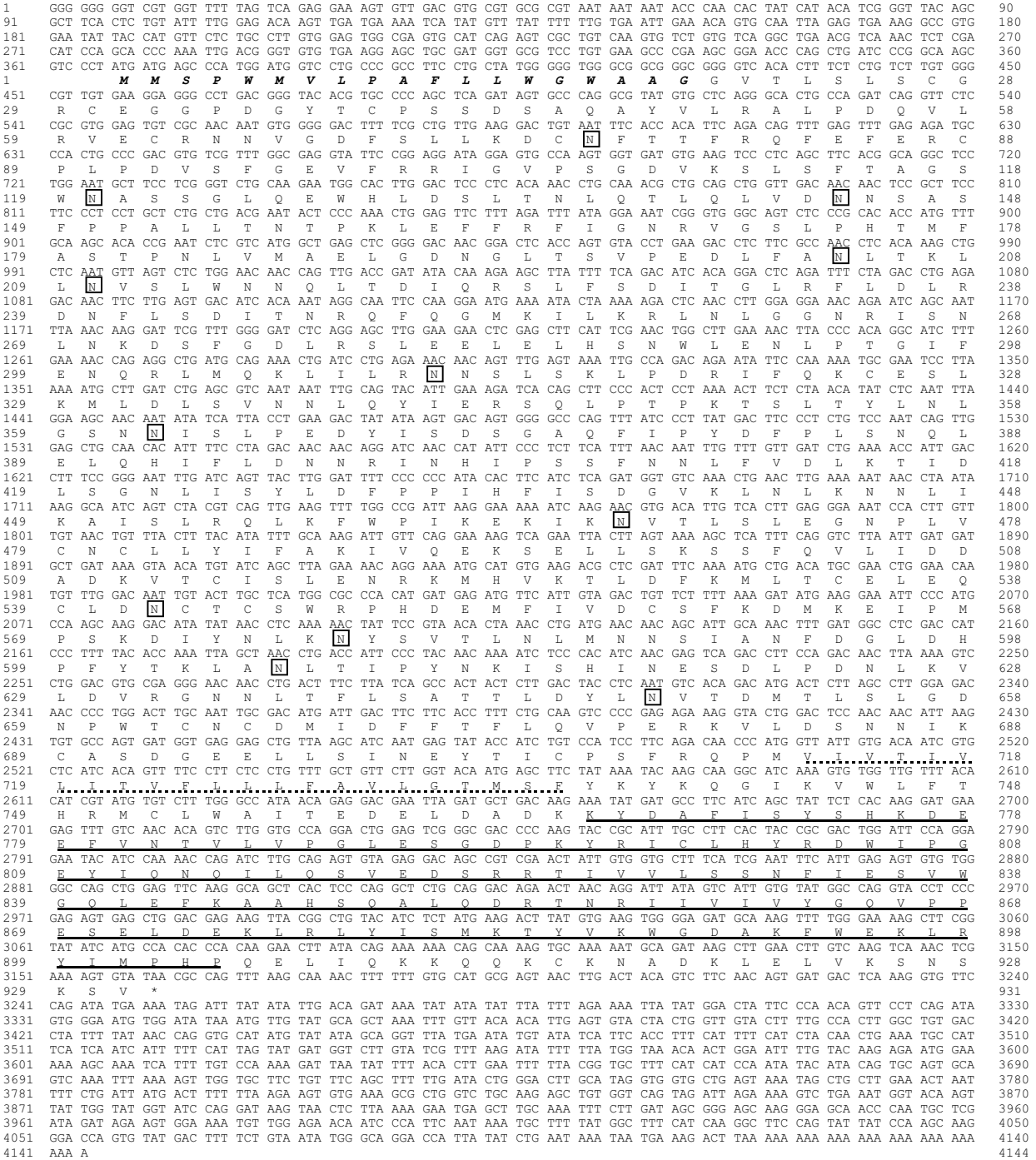


Figure 3. The full-length cDNA sequence and deduced amino acid sequence of PmToll from *P. monodon.* The result of amino acid sequence is coded with one-letter underneath the nucleotide sequence. The predicted signal peptide is italicized. The potential N-liked glycosyaltion sites in the extracellular domain are shown in boxes. The transmembrane region is underlined with a dotted line, while the TIR domain is underlined with a continuous line.

The prevailing LRR consensus sequence in TLRs is the 24-residue motif of x-L-x-x-L-x-L-x-x-N-x-Φ-x-x-Φ-x-x-x-x-F-x-x-L-x (Bell et al., 2003), where x refers to any amino acid, Φ is any hydrophobic residue, L and F are frequently replaced by other hydrophobic residues. Alignment of LRRs in PmToll (Figure 4) reveals that 14 tandem LRR repeats exist in PmToll, whereas only 8 LRRs were predicted by the SMART program (Figure 5). All of the LRRs contain the conserved asparagine residue at position 10, while highly conserved leucine residues were found at positions 2, 5 and 7 of each LRR. In addition, an insertion of seven residues was identified in LRR-10. Moreover, alignment of the TIR domain of PmToll protein with other shrimp and arthropod Toll proteins showed a similar structure (Figure 6). The expression of PmToll was investigated in several tissues including gill, haemocyte, heart, hepatopancreas, lymphoid organs, muscle, nerve, pleopod, stomach, testis and ovary, expression was detected in all tissues using multiplex RT-PCR, although an apparently low level of expression was observed in hepatopancreas (Figure 7).

Consensus	XL	XXLXLXXN	XΦXXΦ	XXXXFXX LX	Position
LRR1	NL	QTLQLVDN	NSASF	PPALLTN TP	135-158
LRR2	KL	EFFRFIGN	RVGSL	PHTMFAS TP	159-182
LRR3	NL	VMAELGDN	GLTSV	PEDLFAN LT	183-206
LRR4	KL	LNVSLWNN	QLTDI	QRSLFSD IT	207-230
LRR5	GL	RFLDLRDN	FLSDI	TNRQFQG MK	231-254
LRR6	IL	KRLNLGGN	RISNL	NKDSFGD LR	255-278
LRR7	SL	EELELHSN	WLENL	PTGIFEN QR	279-302
LRR8	LM	QKLILRNN	SLSKL	PDRIFQK CE	303-326
LRR9	SL	KMLDLSVN	NLQYI	ERSQLPT PK	327-350
LRR10	SL	TYLNLGSNNISLPEDYISDS		-GAQFIP YD	352-381
LRR11	EL	QHIFLDNN	RINHI	-PSSFNN LF	389-411
LRR12	DL	KTIDLSGN	LISYL	DFPPIHF IS	413-436
LRR13	GV	-KLNLKNN	LIKAI	SLRQLKFWP IK	438-462
LRR14	NL	KVLDVRGN	NLTFL	SATTLDY LN	625-648

Figure 4. Alignment of leucine-rich repeats (LRRs) in PmToll. LRRs of PmToll are aligned with the 24-residue prevailing LRR consensus sequence of TLRs (Bell et al., 2003). X refers to any amino acid, Φ is any hydrophobic residue, and L and F are frequently replaced by other hydrophobic residues. Residues that are conserved with the consensus sequence are shaded in grey.

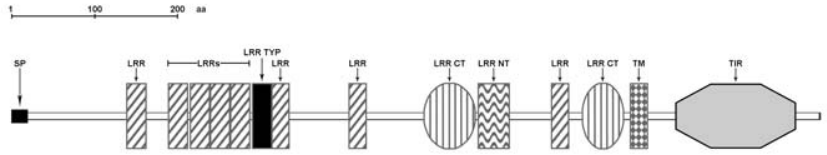


Figure 5. Schematic diagram of PmToll protein predicted by the SMART program. The ectodomain of PmTolls consists of SP, LRR, LRR-CT and LRR-NT. TM is the transmembrane region. The cytosolic domain consists of the TIR/IL1 domain. Abbreviations: SP, signal peptide; LRR, leucine rich reperat; LRR-CT, leucine rich repeat C-terminal domain; LRR-NT, leucine rich repeat N-terminal domain and TIR, Toll/Interleukiin-1R domain.

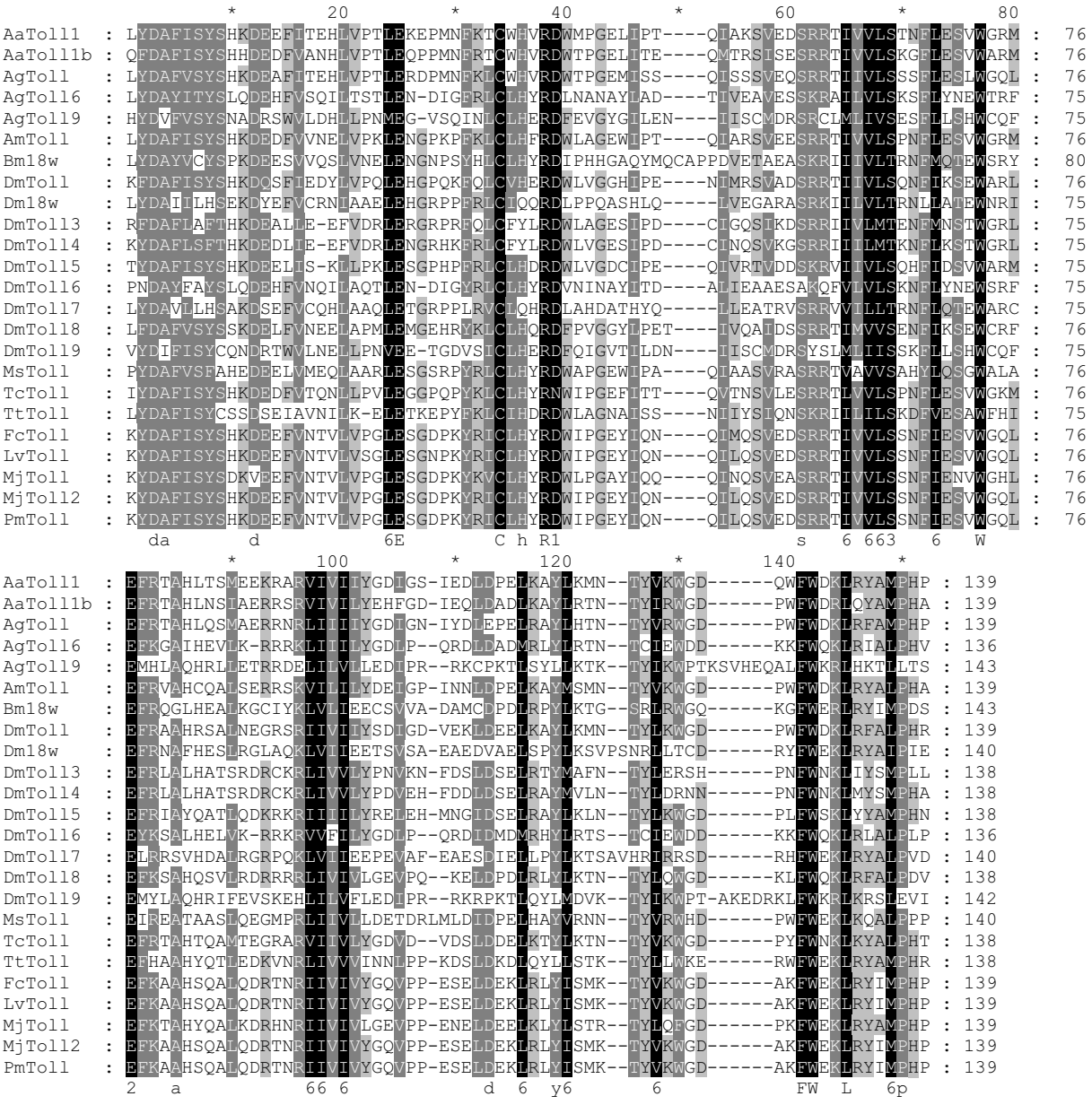


Figure 6. Alignment of TIR domains of Arthropoda Tolls.

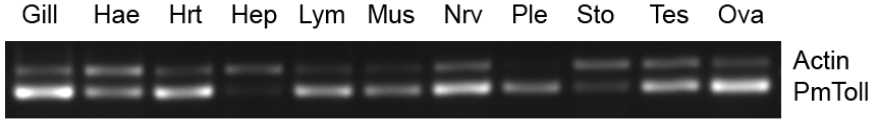


Figure 7. Tissue expression profile of PmToll using multiplex RT-PCR detection. Abbreviations are Hae, Haemocyte; Hrt, Heart; Hep, Hepatopancreas; Lym, Lymphoid organ; Mus, Muscle; Nrv, Nerve; Ple, Pleopod; Sto, Stomach; Tes, Testis and Ova, Ovary.

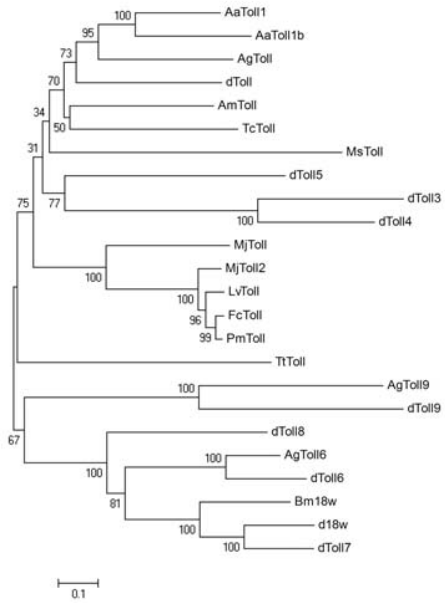


Figure 8. Phylogenetic tree of arthropod TLRs. All full-length amino acid sequences of TLR from Arthropod sequences (Table 2) were aligned and the phylogenetic tree was constructed by using the Bootstrap NJ method with using the program MEGA4.02. The reliability of each branch was tested by 1000 bootstrap replications. Numbers at the branch nodes indicated bootstrap values. The scale bar indicates a branch length of 0.1 amino acid sequence.

The phylogenetic relationship between the deduced amino acid of PmToll and other arthropod Tolls is shown in Figure 8, and the analysis suggests that PmToll is closely related to other shrimp Tolls especially FcToll. Moreover, shrimp Toll proteins are more closely related to DmToll5, DmToll3 and DmToll4 than to other DmTolls. In *F. chinensis*, expression of FcToll in the lymphoid organ has been characterized after bacterial or WSSV challenge, and was shown to have distinct expression profiles. After bacterial challenge FcToll expression was up-regulated whereas FcToll expression after WSSV stimulation was down regulated (Yang et al., 2008). More recently the function of LvToll was studied using an RNAi silencing approach to down regulate expression of LvToll, followed by WSSV or *V. harveyi* challenge. While there was a significant increase in mortality and bacterial CFU counts in LvToll silenced shrimp following *V. harveyi* challenge, there was no difference in mortality rates following WSSV challenge, suggesting that LvToll is an important factor in the shrimp innate immune response to acute *V. harveyi* infection, but not to WSSV (Han-Ching Wang et al., 2010). Similarly, PmToll was not found to be regulated during WSSV challenge of *P. monodon* (Arts et al., 2007). Collectively these results suggest that shrimp Tolls are involved in the innate immune response to bacterial, rather than viral infection and as such further studies elucidating the mechanism of action of Tolls will be of benefit in understanding the mechanism of bacterial pathogenesis of economically important aquatic species.

Table 2. Details of genes used for PmToll analysis.

Species	Name	Accession Number
Aedes aegypti	AaToll1	AAM97775
	AaToll1b	AAM97776
Anopheles gambiae	AgToll	AAL37901
	AgToll6	AAL37902
	AgToll9	AAL37903
Apis mellifera	AmToll	XP_396158
Bombyx mori	Bm18w	BAB85498
Drosophila melanogaster	DmToll	AAQ64935
	Dm18w	AAF57509
	DmToll3	AAF54021
	DmToll4	AAF52747
	DmToll5	AAF86227
	DmToll6	AAF49645
	DmToll7	AAF57514
	DmToll8	AAF49650
	DmToll9	AAF51581
Fenneropenaeus chinensis	FcToll	ABQ59330
Litopenaeus vannamei	LvToll	ABK58729
Manduca sexta	MsToll	ABO21763
Marsupenaeus japonicus	MjToll	BAF99007
	MjToll2	BAG68890
Tribolium castaneum	TcToll	XP_967796
Tachypleus tridentatus	TtToll	BAD12073

References

Alexopoulou L, Holt AC, Medzhitov R and Flavell RA. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor. *Nature.* 413 (6857): 732-738.

Arts JA, Cornelissen FH, Cijsouw T, Hermsen T, et al. (2007). Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol.* 23: 504-513.

Assavalapsakul W. (2007). Important shrimp viruses in Thai shrimp-farming industry : Yellow head virus, White spot syndrome virus and Taura syndrome virus. *J Sci Res Chula Uni (Section T).* 6 (1): 1-8.

Bell JK, Mullen GE, Leifer CA, Mazzoni A, et al. (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* 24: 528-533.

Bendtsen JD, Nielsen H, von Heijne G and Brunak S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340: 783-795.

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, et al. (1993). Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). *Fish Pathology*. 28: 103-109.

Bowie A and O'Neill LA (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J. Leukoc. Biol.* 67: 508-514.

Cerenius L and Soderhall K (2004). The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* 198: 116-126.

Flegel TW (1997). Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotech.* 13: 433-442.

Guillot L, Le Goffic R, Bloch S, Escriou N, et al. (2005). Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem.* 280 (7): 5571-5580.

Han-Ching Wang K, Tseng CW, Lin HY, Chen IT, et al. (2010). RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*. *Dev. Comp. Immunol.* 34: 49-58.

Hoffmann JA. (2003). The immune response of Drosophila. *Nature*. 426 (6962): 33-38.

Inamori K, Ariki S and Kawabata S. (2004). A Toll-like receptor in horseshoe crabs. *Immunol Rev.* 198: 106-115.

Jiravanichpaisal P, Lee BL and Soderhall K (2006). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*. 211: 213-236.

Kawai T and Akira S. (2005). Toll-like receptor downstream signaling. Arthritis Res Ther. 7 (1): 12-19.

Kim CS, Kosuke Z, Nam YK, Kim SK, et al. (2007). Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. *Fish Shellfish Immunol*. 23 (1): 242-246.

Lee SY and Soderhall K (2002). Early events in crustacean innate immunity. *Fish Shellfish Immunol.* 12: 421-437.

Lehnert SA, Wilson KJ, Byrne K and Moore SS. (1999). Tissue-Specific Expressed Sequence Tags from the Black Tiger Shrimp *Penaeus monodon*. *Mar Biotechnol* (NY). 1 (5): 465-476.

Lemaitre B and Hoffmann J (2007). The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* 25: 697-743.

Letunic I, Doerks T and Bork P (2009). SMART 6: recent updates and new developments. *Nucleic Acids Res.* 37: D229-232.

Mekata T, Kono T, Yoshida T, Sakai M , et al. (2008). Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol.* 24: 122-133.

Robalino J, Bartlett T, Shepard E, Prior S, et al. (2005). Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? *J Virol.* 79 (21): 13561-13571.

Robalino J, Bartlett TC, Chapman RW, Gross PS, et al. (2007). Double-stranded RNA and antiviral immunity in marine shrimp: inducible host mechanisms and evidence for the evolution of viral counter-responses. *Dev Comp Immunol.* 31 (6): 539-547.

Rojtinnakorn J, Hirono I, Itami T, Takahashi Y, et al. (2002). Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish Shellfish Immunol.* 13 (1): 69-83.

Schmidt KN, Leung B, Kwong M, Zarember KA, et al. (2004). APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J Immunol*. 172(1): 138-43.

Takeda K and Akira S. (2004a) TLR signaling pathways. *Semin Immunol.* 16 (1): 3-9.

Takeda K and Akira S. (2004b) Microbial recognition by Toll-like receptors. *J Dermatol Sci.* 34 (2): 73-82.

Takeda K and Akira S. (2005) Toll-like receptors in innate immunity. *Int Immunol.* 17 (1): 1-14.

Tamura K, Dudley J, Nei M and Kumar, S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599.

Tirasophon W, Roshorm Y and Panyim S. (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. *Biochem Biophys Res Commun.* 334 (1): 102-107.

Tirasophon W, Yodmuang S, Chinnirunvong W, Plongthongkum N, et al. (2007). Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. *Antiviral Res.* 74 (2): 150-155.

Westenberg M, Heinhuis B, Zuidema D and Vlak JM. (2005). siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. *Virus Res.* 114 (1-2): 133-139.

Xu J, Han F and Zhang X. (2007). Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. *Antiviral Res.* 73 (2): 126-131.

Yang C, Zhang J, Li F, Ma H, et al. (2008). A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol.* 24**:** 564-574.

Yang LS, Yin ZX, Liao JX, Huang XD, et al. (2007). A Toll receptor in shrimp. *Mol. Immunol.* 44**:** 1999-2008.

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, et al. (2006). YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochem Biophys Res Commun*. 341 (2): 351-356.

Output ที่ได้จากโครงการ

ผลงานวิจัยที่ตีพิมพ์ลงในวารสารวิชาการระดับนานาชาติ จำนวน 2 บทความ

Assavalapsakul W, Chinnirunwong W and Panyim S. (2009). Application of YHV-protease dsRNA for protection and therapeutic treatment against Yellow Head virus infection in *Litopenaeus vannamei*. *Dis Aquat Organ*. 84 (2): 167-171.

Assavalapsakul W and Panyim S. Molecular cloning and tissue distribution of Toll receptor, PmToll gene from black tiger shrimp, *Penaeus monodon*. *Genet Mol Res.* (Accepted).

ผลงานวิจัยที่ตีพิมพ์ลงในวารสารวิชาการระดับชาติ จำนวน 1 บทความ

Poramate Jiaranai, Sakol Panyim, Wanchai Assavalapsakul. Molecular cloning and characterization of Toll receptor in *Penaeus monodon*. Proceeding of the 7th National Symposium on Marine Shrimp 2010 : page 81-89.

ภาคผนวก

1. บทความลงในวารสารวิชาการระดับนานาชาติ จำนวน 2 บทความ ดังนี้ :

Assavalapsakul W, Chinnirunwong W and Panyim S. (2009). Application of YHV-protease dsRNA for protection and therapeutic treatment against Yellow Head virus infection in *Litopenaeus vannamei*. *Dis Aquat Organ*. 84 (2): 167-171.

Assavalapsakul W and Panyim S. Molecular cloning and tissue distribution of Toll receptor, PmToll gene from black tiger shrimp, *Penaeus monodon*. *Genet Mol Res.* (Accepted).

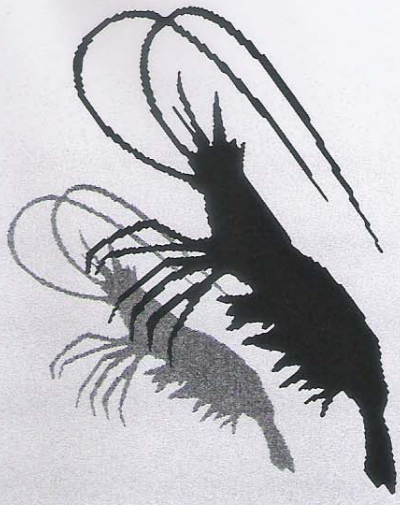
2. ผลงานบางส่วนไปเสนอในการประชุมวิชาการกุ้งทะเลแห่งชาติ ครั้งที่ 7 “คุณภาพกุ้งไทยสู่อาหารปลอดภัยระดับโลก” ในวันที่ 7-8 กันยายน 2553 ณ โรงแรมทวินโลตัส จังหวัดนครศรีธรรมราช (Proceeding of the 7th National Symposium on Marine Shrimp) และทำการตีพิมพ์ลงใน Proceeding of the 7th National Symposium on Marine Shrimp 2010 จำนวน 1 บทความ ดังนี้ :

Poramate Jiaranai, Sakol Panyim, Wanchai Assavalapsakul. Molecular cloning and characterization of Toll receptor in *Penaeus monodon*. Proceeding of the 7th National Symposium on Marine Shrimp 2010 : page 81-89.

Proceedings

การประชุมวิชาการกุ้งทะเลแห่งชาติ ครั้งที่ 7

The 7th National Symposium on Marine Shrimp



เรื่อง คุณภาพกุ้งไทย สู่อาหารปลอดภัยระดับโลก

วันที่ 7 – 8 กันยายน 2553

โรงแรมทวินโลตัส จังหวัดนครศรีธรรมราช

BIOTEC a member of NSTDA สวทช NSTDA

SHRIMP RESEARCH UNIT WALAILAK UNIVERSITY

การประชุมวิชาการกุ้งทะเลแห่งชาติ ครั้งที่ 7
"คุณภาพกุ้งไทย สู่อาหารปลอดภัยระดับโลก"

วันที่ 7-8 กันยายน 2553
โรงแรมทวินโลตัส จังหวัดนครศรีธรรมราช

วันอังคารที่ 7 กันยายน 2553

เวลา	สถานที่	
	ห้องประชุมบงกชรัตน์	ห้องประชุมไทยทักษิณ
08.00 – 09.00	ลงทะเบียน	
09.00 – 09.15	พิธีเปิด	
09.15 – 09.45	**การบรรยายพิเศษ เรื่อง งานวิจัยด้านกุ้ง และแผนงานในอนาคต** โดย ศ.เกียรติคุณ ดร. มรกต ตันติเจริญ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ	
09.45 – 10.15	**การบรรยายพิเศษ เรื่อง สถานภาพงานวิจัยด้านระบบภูมิคุ้มกันในกุ้ง** โดย ศ.ดร. อัญชลี ทัศนาขจร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	
10.15 – 10.45	**การบรรยายพิเศษ เรื่อง สถานภาพงานวิจัยด้านโรคกุ้งและการตรวจวินิจฉัย** โดย ศ.ดร. ทิมโมที เฟลเกล คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	
10.45 – 11.00	รับประทานอาหารว่าง	
11.00 – 11.30	**การบรรยายพิเศษ เรื่อง สถานภาพงานวิจัยด้านฮอร์โมนและระบบสืบพันธุ์** โดย ดร. ศิราวุธ กลิ่นบุหงา ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ	
11.30 – 12.00	**การบรรยายพิเศษ เรื่อง สถานภาพงานวิจัยด้านการเลี้ยงและสิ่งแวดล้อมในบ่อกุ้ง** โดย รศ.ดร. ชลอ ลิ้มสุวรรณ คณะประมง มหาวิทยาลัยเกษตรศาสตร์	
12.00 – 14.00	รับประทานอาหารกลางวัน และนำเสนอผลงานภาคโปสเตอร์	
	การนำเสนอผลงานภาคบรรยาย	
	Shrimp diseases and detections	**Genomics and proteomics**
14.00 – 14.20	**OP-01** **Yellow head virus (YHV) transmission risk from commodity shrimp is reduced to negligible levels by normal processing** Kallaya Sritunyalucksana	**OP-17** **Proteome components of Penaeus shrimps as analyzed from combined ESTs and cDNAs from multiple species** Burachai Sonthayanon
14.20 – 14.40	**OP-02** **Identification of hemocyte populations involved in viral infection of the freshwater prawn, *Macrobrachium rosenbergiiuthair*** Tanatchaporn Utairungsee	**OP-18** **Molecular characterization and expression analysis of *Calnexin* under heat stress in the giant tiger shrimp (*Penaeus monodon*)** Virak Visudtiphole

เวลา	สถานที่	
	ห้องประชุมบงกชรัตน์	ห้องประชุมไทยทักษิณ
14.40 – 15.00	**OP-03** **Identification of a hemocyte population involved in WSSV-persistent infection in a mud crab (*Scylla olivacea*)** Piyachat Sa-nguanrut	**Genetic improvement** **OP-19** **Genetic diversification of banana shrimp populations by using *Penaeus monodon*'s microsatellite marker** Kittisak Chawawisit
15.00 – 15.20	**OP-04** **Genome characterization of Laem Singh virus, a new virus found in *Penaeus monodon*** Jiraporn Srisala	**OP-20** **Characterization of candidate genes involved in growth of black tiger shrimp, *Penaeus monodon*** Amornrat Tangprasittipap
15.20 – 15.40	รับประทานอาหารว่าง	
15.40 – 16.00	**OP-05** ***Macrobrachium rosenbergii* nodavirus (*Mr*NV) associated with white muscle disease of *Penaeus vannamei*** Saengchan Senapin	**OP-21** **Genetic parameter estimates for growth in the black tiger shrimp *(Penaeus monodon* Fabricius) rearing in closed-system concrete tank** Opor Siwasutham
16.00 – 16.20	**OP-06** **Application of high resolution melt (HRM) analysis for duplex detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in shrimp** Sudkhate Molthathong	**OP-22** **Identification and expression analysis of *insulin degrading enzyme* (*IDE*) in the giant tiger shrimp *Penaeus monodon*** Bavornlak Khamnamtong
16.20 – 16.40	**OP-07** **Development of loop-mediated isothermal amplification (LAMP) combining with lateral flow dipstick (LFD) and a simple turbidimeter for detection of shrimp viruses** Wansika Kiatpathomchai	
16.40 – 17.00	**OP-08** **Effect of salinities on stability of MBV (Monodon baculovirus)** Supaporn Hnuchu	

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เวลา	สถานที่	
	ห้องประชุมบงกชรัตน์	ห้องประชุมไทยทักษิณ
	Shrimp diseases and detections	**Reproductive system**
09.00 – 09.20	**OP-09** **Autonomous, genetic modification of shrimp in response to viral pathogens** Timothy W. Flegel	**OP-23** **Expression analysis of heat shock protein genes in different ovarian stages of black tiger shrimp (*Penaeus monodon*)** Wanilada Rungrassamee
09.20 – 09.40	**OP-10** **Toxicity of dissolved organic nitrogen on black tiger shrimp and relation of white spot infection** Suntipon Pumcong	**OP-24** **A new strategy to stimulate ovarian maturation in female *Penaeus monodon* broodstock** Supattra Treerattrakool

เวลา	สถานที่	
	ห้องประชุมบงกชรัตน์	ห้องประชุมไทยทักษิณ
09.40 – 10.00	**OP-11** **Identification and characterization of differentially expressed genes in shrimp (*Penaeus vannamei*) infected with yellow head virus (YHV)** Kingkamon Junkunlo	**OP-25** **Isolation and characterization of *MEK* and its transcriptional profile during ovarian development of the giant tiger shrimp *Penaeus monodon*** Pattareeya Ponza
10.00 – 10.20	**OP-12** **Identification of candidate genes potentially involved in disease resistance in *Litopenaeus vannamei*** Siriporn Pongsomboon	**OP-26** **Transcriptomic comparison between wild and domesticated female black tiger shrimp (*Panaeus monodon*)** Umaporn Uawisetwathana
10.20 – 10.40	รับประทานอาหารว่าง	**OP-27** **Identification of genes involved in testicular development in male black tiger shrimp (*Penaeus monodon*) using cDNA microarray analysis** Rungnapa Leelatanawit
10.40 – 11.00	**OP-13** **Autonomous shrimp genetic modification for production of viral antisense RNA** Heny Budi Utari	รับประทานอาหารว่าง
11.00 – 11.20	**OP-14** **RNAi for inhibition of *Penaeus monodon* densovirus (*Pm*DNV) replication in shrimp** Pongsopee Attasart	
11.20 – 11.40	**OP-15** **Double-stranded RNA-mediated inhibition of shrimp viral gene in *Spodoptera frugiperda* (Sf9) cells** Gatesara Theerawanitchpan	
11.40 – 12.00	**OP-16** **Roles of *N*-linked glycosylation on yellow head virus (YHV) replication, maturation and pathogenesis** Chumporn Soowannayan	
12.00 – 14.00	รับประทานอาหารกลางวัน และนำเสนอผลงานภาคโปสเตอร์	
	Immune system	**Feed and nutrition**
14.00 – 14.20	**OP-28** **Identification of binding partners of alpha-2-macroglobulin in the black tiger shrimp using yeast two-hybrid screening** Vorrapon Chaikeeratisak	**OP-36** **Comparison of using various sizes of artificial sand (Vermiculite™) and natural sand for the culture of sand worm (*Perinereis nuntia*)** Thanya Duangchinda
14.20 – 14.40	**OP-29** **Molecular cloning and characterization of Toll receptor in *Penaeus monodon*** Wanchai Assavalapsakul	**OP-37** **Optimal dietary protein level for the culture of sand worm (*Perinereis nuntia*)** Thanya Duangchinda
14.40 – 15.00	**OP-30** **Application of RNA interference to study the gene function in shrimp prophenoloxidase system** Piti Amparyup	**OP-38** **Effects of different culture medium on growth and biochemical composition of *Chaetoceros gracilis* for nursing juvenile shrimp (*Penaeus monodon*)** Pornpimol Pimolrat

เวลา	สถานที่	
	ห้องประชุมบงกชรัตน์	ห้องประชุมไทยทักษิณ
15.00 – 15.20	**OP-31** **Efficacy of oil macerated garlic extract on immune responses and diseases resistance in white shrimp (*Litopeneaus vannamei* Boone)** Jumroensri Thawonsuwan	**OP-39** **Effect of *Chaetoceros gracilis* and oyster on sperm quality of black tiger shrimp** Satit Songtuay
15.20 – 15.40	รับประทานอาหารว่าง	
15.40 – 16.00	**OP-32** **Effects of *Lactobacillus* spp. on inhibit of white spot syndrome virus (WSSV)** Supannee Suwanpakdee	**OP-40** **Development of commercial prototype continuous culture system for diatom (*Chaetoceros* sp.) cultivation in shrimp hatchery** Paveena Tapaneeyaworawong
16.00 – 16.20	**OP-33** **Preparation of chitosan nanoparticles-biosubstants for shrimp** Jaturong Matidtor	**Pond management and environment** **OP-41** **Effects of water-recirculation on the performance of integrated aquaculture in treating of shrimp-pond effluent** Shewin Attasat
16.20 – 16.40	**OP-34** **Bacterial community associated with the intestinal tract of juvenile black tiger shrimp in rearing ponds** Sage Chaiyapechara	**OP-42** **Effect of nitrification biofilter on water quality and production yield of Pacific white shrimp in outdoor tanks** Sorawit Powtongsook
16.40 – 17.00	**OP-35** **Knocking down a Taura syndrome virus (TSV) binding protein Lamr is lethal for the whiteleg shrimp *Penaeus vannamei*** kornsunee phiwsaiya	**OP-43** **Rearing of *Penaeus monodon* in closed culture system in concrete tanks equipped with sludge removal devices** Kritsawat Nganing
17.00	ปิดการประชุม	

OP-29

Molecular cloning and characterization of Toll receptor in *Penaeus monodon*

การโคลนและสมบัติของตัวรับโทลล์ในกุ้งกุลาดำ

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Abstract:
Black tiger shrimp is one of the economic aquatic organisms which brings a lot of income to Thailand; however, the black tiger shrimp farming industry has decreased because of the susceptibility of the shrimp to many kinds of shrimp pathogens such as white spot syndrome virus and yellow head virus. An understanding of the shrimp immune system is key to inhibiting and/or controlling diseases and epidemics. RNA interference is part of the shrimp immune system protecting it from pathogen infection. The hypothesis of this work is that the Toll receptor is involved in RNA interference and the expression of the immune gene systems. The Toll gene from the black tiger shrimp was cloned and sequenced using 5′ and 3′ Rapid Amplification cDNA End methods. The result showed that the full-length cDNA Toll gene has 4,134 nucleotides which encode 931 amino acids. The Toll protein contains the distinct structure/functional motif of the Toll-like receptor (TLR) family, including an extracellular domain containing 13 leucine-rich repeats (LRRs) flanked by cysteine-rich motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain. The specific region of the PmToll receptor was used to design and construct the stem-loop of a dsRNA-PmToll expression cassette for expression of the dsRNA-PmToll, which is injected into shrimp to withhold the PmToll expression. The results showed that the PmToll receptor gene could be withheld within 24 – 72 hours after dsRNA injection. Further experimentation is necessary to establish whether dsRNA can be entered into shrimp after the withholding of the PmToll receptor and to study the expression level of immune genes after the withholding of the PmToll receptor gene, following pathogen infection.

Keywords: Black tiger shrimp, *Penaeus monodon*, Toll receptor, RNA interference (RNAi), Double-stranded RNA (dsRNA)

บทคัดย่อ:
กุ้งกุลาดำเป็นสัตว์น้ำเศรษฐกิจของประเทศไทยที่สามารถนำรายได้เข้าสู่ประเทศไทยเป็นจำนวนมาก อย่างไรก็ตามการเลี้ยงกุ้งกุลาดำมีจำนวนลดน้อยลง เนื่องมาจากกุ้งกุลาดำสามารถที่จะเกิดโรคจากการติดเชื้อจุลชีพต่าง ๆ ได้ง่าย อาทิเช่น ไวรัสตัวแดงดวงขาว ไวรัสหัวเหลือง เป็นต้น การเข้าใจระบบภูมิคุ้มกันในกุ้งกุลาดำถือเป็นสิ่งจำเป็นที่จะช่วยในการยับยั้ง และ/หรือ ควบคุมการระบาดของโรคได้ กระบวนการยับยั้งระดับอาร์เอ็นเอเป็นกระบวนการหนึ่งในระบบภูมิคุ้มกันของกุ้งที่จะป้องกันการติดเชื้อจุลชีพ สมมติฐานของงานวิจัยนี้คือ ตัวรับโทลล์เกี่ยวข้องกับกระบวนการยับยั้งการแสดงออกระดับอาร์เอ็นเอ และการแสดงออกของยีนระบบภูมิคุ้มกัน งานวิจัยนี้ได้ทำการโคลนตัวรับโทลล์จากกุ้งกุลาดำ ด้วยวิธีการ 5′ และ 3′ Rapid Amplification cDNA End พบว่ายีนตัวรับโทลล์ มีขนาด 4,134 นิวคลีโอไทด์ แปลรหัสได้ 931 กรดอะมิโน ซึ่งประกอบด้วยบริเวณอนุรักษ์ของตัวรับโทลล์ กล่าวคือ โปรตีนที่อยู่ภายนอกเซลล์ มี leucine-rich repeat (LRRs) 13 ตำแหน่ง และมี cysteine-rich motifs แทรกอยู่ และโปรตีนที่อยู่ภายในเซลล์บริเวณ Toll/interleukin-1 receptor (TIR) ลำดับนิวคลีโอไทด์ของตัวรับโทลล์ที่มีความจำเพาะถูกนำมาใช้ในการสร้างพลาสมิดที่มีการแสดงออกของอาร์เอ็นเอสายคู่ของตัวรับโทลล์ เพื่อที่จะใช้อาร์เอ็นเอสายคู่ดังกล่าวฉีดเข้ากุ้ง เพื่อยับยั้งการแสดงออก

ของตัวรับโทลล์ ผลการทดลองพบว่า ตัวรับโทลล์สามารถถูกยับยั้งการแสดงออกได้ 24 – 72 ชั่วโมง หลังจากการฉีดอาร์เอ็นเอสายคู่ ในการทดลองขั้นต่อไป คือ ทดสอบการนำเข้าอาร์เอ็นเอ สายคู่ว่าสามารถถูกนำเข้าได้หรือไม่ หลังจากยับยั้งการแสดงออกของตัวรับโทลล์ และศึกษาระดับการแสดงออกของยีนระบบภูมคุ้มกันหลังจากยับยั้งการแสดงออกของตัวรับโทลล์และตามด้วยการติดเชื้อจุลชีพ

คำสำคัญ: กุ้งกุลาดำ, ตัวรับโทลล์, การยับยั้งการแสดงออกระดับอาร์เอ็นเอ, อาร์เอ็นเอสายคู่

Introduction:

Black tiger shrimp is one of the economic aquatic organisms which brings a lot of income to Thailand; however, the black tiger shrimp farming industry has decreased because of the susceptibility of the shrimp to many kinds of shrimp pathogens such as white spot syndrome virus and yellow head virus. An understanding of the shrimp immune system is key to inhibiting and/or controlling diseases and epidemics. The shrimp immune system, as in other invertebrate species, depends mainly on innate immunity, which can be divided into humoral defenses (activation of various proteolytic cascades such as the prophenoloxidase [proPO] system, hemolymph clotting mechanism, melanization, and antimicrobial immune response) and cellular defenses (phagocytosis, encapsulation, cellular degranulation, and the release of defense factors) (Lee SY and Söderhäll K, 2002 ; Cerenius L and Söderhäll K, 2004 ; Jiravanichpaisal P *et al.*, 2006; Han-Ching Wang K *et al.*, 2010). The innate immune responses are triggered when cellular or plasma pattern recognition receptors (PRRs) recognize and bind to their specific pathogen-associated molecular patterns (PAMPs). Although there is not yet any direct evidence of these mechanisms in shrimp, it is reasonable to expect that shrimp hemocytes might also use PRRs to regulate down-stream gene expression in the same way (Han-Ching Wang K *et al.*, 2010). Additionally, in *Drosophila*, the down-stream gene expression regulated by the Toll and immune deficiency (Imd) pathways are important effectors in innate immunity (Wang PH *et al.*, 2009). However, the molecular ligand-recognition patterns and identification of these penaeid Toll classes remain unknown (Mekata T *et al.*, 2008).

The hypothesis of this work is that the PmToll receptor is involved in RNA interference and the expression of the immune gene systems. The Toll cDNA of black tiger shrimp has been cloned and characterized to establish whether dsRNA can be entered into shrimp after the withholding of the PmToll receptor and to study the expression level of immune genes after withholding of the PmToll receptor gene, following pathogen infection.

Materials and Methodologies:

Black Tiger Shrimp

The shrimps used in this study were purchased from a commercial farm in the domestic area and reared in sea water tank system with a salinity of 10 parts per thousand (ppt) at 25 – 28 °C for 7 days before the experiments.

Total RNA isolation and cDNA synthesis

The shrimp's tissues were collected in TRI Reagent (Molecular Research Center) and stored at -80 °C. Subsequently, total RNA was then extracted using TRI Reagent and first-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (Fermentas) with oilgo dT primer, random hexanucleotide primer, or specific primer.

Cloning and sequencing of PmToll cDNA fragment

To synthesize the first-strand cDNAs, five micrograms of total RNA was subjected to reverse transcription using M-MLV Reverse Transcription System (Fermentas) according to the supplied procedure. PCR was performed using the cDNA as prepared above with PRT primer (Table 1) which amplifies the initial sequence by using Toll RDW and PM1 primers. Having isolated this partial PmToll receptor, the entire length sequence was obtained using a 5′-RACE-PCR (Fig. 1) with the gene-specific primers shown in Table 1. All PCR reactions were performed using the following: 10× *Taq* Buffer 2.5 µL, 2 µL dNTPs (2 mM of each), 0.5 µL *Taq* polymerase (5 U/µL; Fermentas), 1 µL of each gene-specific primer (10 µM), template cDNA 2 µL and 16 µL distilled

water. The PCR conditions comprised 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 – 55 °C for 30 s and 72 °C for 1.5 min, followed by an extension of 72 °C for 10 min. The products were cloned into the pGEM-T Easy vector (Promega) and transformed into DH5α. DNA from at least five independent clones was extracted using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced using 1st BASE sequencing unit (Malaysia).

Sequence analysis of PmToll

The nucleotide sequence and deduced amino acid sequence of PmToll cDNA were analyzed using the BLAST algorithm (NCBI, http://ncbi.nlm.nih.gov/BLAST). The signal peptide, extracellular domain, transmembrane, cytoplasmic domains, and other characteristics of PmToll were predicted by the simple modular architecture research tool (SMART) program (http://smart.embl-heidelberg.de/). Potential N-linked glycosylation sites were predicted by NetNGlyc 1.0 Serve (http://www.cbs.dtu.dk/services/NetNGlyc/).

Stem-loop dsRNA PmToll plasmid construction, bacterial induction and dsRNA purification

Recombinant plasmid expression stem-loop PmToll RNA was constructed in pET17b (Novagen). A 250 bp cDNA fragment of PmToll was amplified using specific primers PmToll-S-Xba I and PmToll-S-EcoRI-XhoI (Table 1). The first PCR fragment was cloned into pET17b in *Xba* I and *Xho* I site. The first recombinant plasmid was digested with *Eco* RI and *Xho* I site, then ligated with 200 bp cDNA fragment, which is the reverse direction of the previous PmToll, was amplified using specific primers PmToll-A-EcoRI and PmToll-A-XhoI (Table 1), then ligated into the *Eco* RI and *Xho* I sites. Therefore, the pET17b plasmid containing the entire 450 bp insert of the PmToll-S with extra sequence (loop, 50 bp) and the PmToll-A was obtained and named pET-dsRNA-PmToll. The PCR was performed in the total volume of 25 µl composed of 2 µl of cDNA template, 10 pmol of each primer pair, 2 mM (each) dNTPs, and 0.9 U of *Vent* DNA polymerase (NEB) in PCR buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (vol/vol) Triton X-100]. The PCR conditions were as follows; 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by the extension of 72 °C for 10 min. Then, pET-dsRNA-PmToll was transformed into the HT115 bacterial host, a RNase III deficient *E. coli* strain. The methodologies for dsRNA induction and dsRNA purification were based on Ongvarrasopone C *et al.*, (2007).

Knock-down of PmToll *in vivo* expression by dsRNA-mediated RNA interference

For gene knock-down experiments, the experimental group (weight 4 – 5 g/shrimp) was injected with dsRNA-PmToll (2.5 µg/g shrimp) into hemolymph using 1 ml syringe with 29 gauge needle while the control groups were injected with dsRNA-GFP (Yodmuang S *et al.*, 2005) or 150 mM NaCl only. To determine the earliest time point of maximal silencing, gill samples from 3 shrimps of each treatment were collected at day 1, 2, 3, 4 and 5 post-dsRNA injection and total RNA was extracted. RNA was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Fermentas) with PRT primer according to the manufacturer's instructions.

Semi-quantitative PCR

To determine the relative amount of PmToll gene in the samples, PCR of an internal control gene (shrimp beta actin) was included. Semi-quantitative PCR was performed using the cDNA as prepared above which amplifies the PmToll gene and shrimp beta actin by using PmToll F and PmToll R primers and actin F and actin R primers, respectively (Table 1). The temperature profile for PCR conditions were as follows; 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. After 30 cycles, the reaction was held at 72 °C for another 10 min.

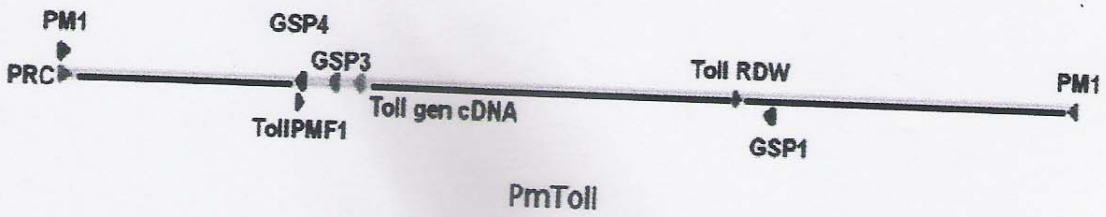

Figure 1 Schematic representation of PmToll cDNA. PmToll cDNA is shown and the positions of the primers are indicated

Table 1 PCR primers

For cloning of PmToll cDNA and 5′-RACE-PCR		
	PRT primer	5'-CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTT-3'
	PM1 primer	5'-CCGGAATTCAAGCTTCTAGAGGATCCTT-3′
	Toll RDW	5′-TGCCTTCACTACCGCGACTGG-3′
	GSP1	5'-AGCCTGGGAGTGAGCTGC-3'
	Toll PMF1	5'-AGTGTACCTGAAGACCTCTT-3'
	Toll gen cDNA	5'-GAGTTCTTCCAAGCTCCTGAGATC-3'
	GSP3	5'-GCCTATTTGTGATGTCACTC-3'
	GSP4	5'-GCGAAGAGGTCTTCAGGTACACT-3'
	PRC primer	5'-CCGGAATTCAAGCTTCTAGAGGATCCTTGGGGGGGGGGGGGGG-3'
For stem-loop dsRNA PmToll Plasmid construction		
	PmToll-S-XbaI	5′-TCTAGAGATCTGAAAACCAATGAC-3′
	PmToll-S-EcoRI-XhoI	5′-CTCGAGGGGGGAATTCCTTTTCCTGAACAATCTTTGC-3′
	PmToll-A-EcoRI	5′-CTCGAGGATCTGAAAACCAATGAC-3′
	PmToll-A-XhoI	5′-GAATTCTCCCTCAAGTGACAATG-3′
For semi-quantitative PCR of PmToll and shrimp beta actin		
	PmToll F	5′-GTCCAATCAGTTGGAGCTGC-3′
	PmToll R	5′-GAAATCGAGCGTCTTCACATGC-3′
	Actin F	5′-GACTCGTACGTGGGCGACGAGG-3′
	Actin R	5'-AGCAGCGGTGGTCATCTCCTGCTC-3'

Results and Discussion:

Host defense in shrimp is believed to rely largely on innate immunity (Loker ES *et al.*, 2004). Innate immunity is a sensitive non-self recognition system triggered by the components of pathogens, called pathogen-associated molecular pattern (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acid, and non-methylated CpG DNA (Hoffmann JA *et al.*, 1999; Söderhäll K and Cerenius L, 1998). PAMPs are recognized by a set of germline-encoded receptors referred to as pattern-recognition receptors (PRRs). Tolls and Toll-like receptors (TLRs) have been recognized as major PRRs and they play an essential role in recognition of microbes during host defense (Akira S *et al.*, 2001, 2006 ; Lemaitire B *et al.*, 1996; Medzhitov R *et al.*, 1997). TLRs are evolutionarily conserved transmembrane glycoproteins characterized by an extracellular domain containing various numbers of leucine-rich repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie A and O'Neill LAJ, 2000). Although quiet recently

truncated PmToll, full-length of LvToll, MjToll and FcToll from *Penaeus monodon* (Arts JA *et al.*, 2007), *Litopenaeus vannamei* (Yang LS *et al.*, 2007), *Marsupenaeus japonicus* (Mekata T *et al.*, 2008) and *Fenneropenaeus chinensis* (Yang C *et al.*, 2008), respectively, have been cloned, some of their functions in shrimp innate immunity against foreign molecules have been studied. However, in this study, we first reported the identification of a full-length Toll receptor in black tiger shrimp, *P. monodon.* The cloned full-length sequences of the PmToll cDNA consisting of 4,134 nucleotides containing 5'-UTR of 357 nucleotides, 3'-terminal UTR of 811 nucleotides with a classical polyadenylation siganl sequence AATAAA and a poly A-tail of 26 nucleotides, and an open reading frame coding for 931 amino acid (Fig. 2). The deduced amino acid sequence of PmToll is a typical type I membrane domain protein, characteristic of TLRs' functional domains. It includes a putative signal peptide (residues 1-19), an extracellular domain (residues 133-706) consisting of leucine-rich repeats (LRRs) flanked by cysteine-rich motifs, a single-pass transmembrane portion (residues 713-735) and a cytoplasmic TIR domain (residues 766-904). Twelve potential N-linked glycosylation sites, which were predicted by NetNGlyc 1.0 Server, are deductively located in the ectodomain. Finally, many structural features are conserved in the regions that flank the LRRs, including all of 18 cysteine residues in the LRR-CT and LRR-NT regions, and the sequence NPXXC(N/D)C, in the two LRR-CT regions of PmToll. The prevailing LRR consensus sequence in TLRs is the 24-residue motif of x-L-x-x-L-x-L-x-x-N-x-Φ-x-x-Φ-x-x-x-x-F-x-x-L-x (Bell JK *et al.*, 2003), where x refers to any amino acid, Φ is any hydrophobic residue, L and F are frequently replaced by other hydrophobic residues. Alignment of LRRs in PmToll (Fig. 3) reveals that 13 tandem LRR repeats exist in PmToll. All of them contain the significantly conserved asparagine residue at position 10, while highly conserved leucine residues were found at position 2, 5 and 7 of each LRR. In addition, an insertion of seven residues was identified in LRR-10. Moreover, the PmToll protein was closely similar to other shrimp's Toll Proteins (Fig. 4).

Addtionally, the functions of shrimp's Tolls have been studied. In *F. chinensis*, FcToll has been characterized and showed that, after bacterial or WSSV challenge, the expression of FcToll in lymphoid organ showed distinct profiles. The up-regulated FcToll expression after bacterial challenge indicated that it may be inducible and participate in shrimp innate immune responses, whereas, the FcToll expression after WSSV stimulation was downregulated (Yang C *et al.*, 2008). In *M. japonicus,* the expression profile of MjToll gene was studied and showed that it was increased by stimulation with 10 mg/mL of PG at 9 and 12 h, and its levels were 26.8-and 76.0-fold higher compared to the control. Its similar function in TLR2 in humans plays a major role in detecting Gram-positive bacteria and is also involved in the recognition of other microbial components such as LPS from Gram-negative bacteria or lipoteichoic acid from Gram-positive bacteria (Mekata T *et al.*, 2008). Finally, PmToll has also been identified and found that there is no regulation during the first 24 hpi during a WSSV challenge suggesting that PmToll is not regulated in time during a WSSV challenge, not only in the first 24 h, but also due to the rapidity of the innate immune system, not in a later stage of infection (Arts JA *et al.*, 2007).

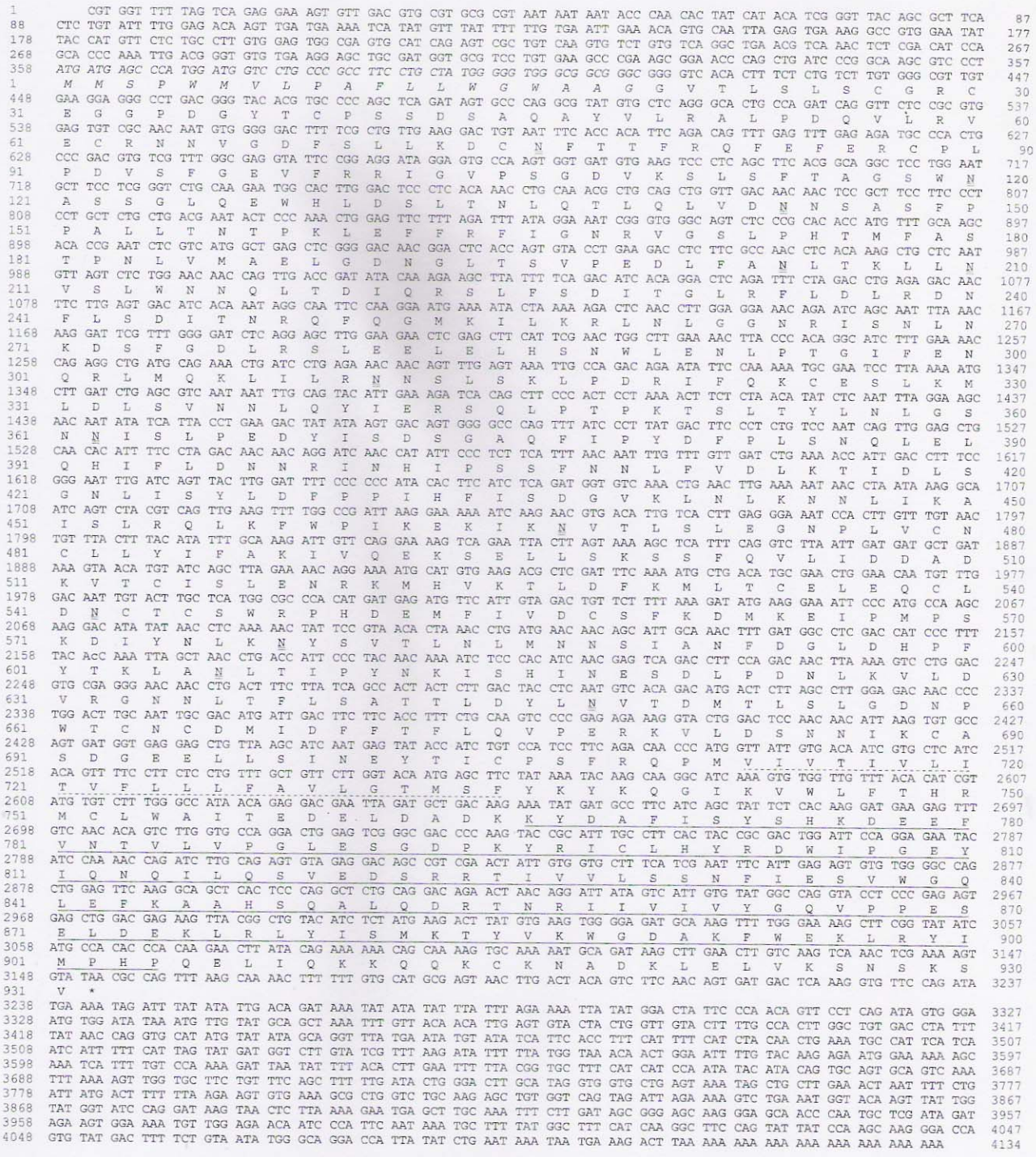

Figure 2 The nucleotide sequence and the deduced amino acid sequence of the PmToll gene from *P. monodon.* The result of amino acid sequence is coded with one-letter underneath the nucleotide sequence. The predicted signal peptide is italicized. The potential N-liked glycosyaltion sites in the extracellular domain are shown in double lines. The establishment of transmembrane region is presented in a dotted line while the TIR domain is underlined.

Consensus	XL	XXLXLXXN	XΦXXΦ	XXXXFXX LX	Position
LRR1	NL	QTLQLVDN	NSASF	PPALLTN TP	135-158
LRR2	KL	EFFRFIGN	RVGSL	PHTMFAS TP	159-182
LRR3	NL	VMAELGDN	GLTSV	PEDLFAN LT	183-206
LRR4	KL	LNVSLWNN	QLTDI	QRSLFSD IT	207-230
LRR5	GL	RFLDLRDN	FLSDI	TNRQFQG MK	231-254
LRR6	IL	KRLNLGGN	RISNL	NKDSFGD LR	255-278
LRR7	SL	EELELHSN	WLENL	PTGIFEN QR	279-302
LRR8	LM	QKLILRNN	SLSKL	PDRIFQK CE	303-326
LRR9	SL	KMLDLSVN	NLQYI	ERSQLPT PK	327-350
LRR10	SL	TYLNLGSNNISLPEDYISDS		-GAQFIP YD	352-381
LRR11	EL	QHIFLDNN	RINHI	-PSSFNN LF	389-411
LRR12	DL	KTIDLSGN	LISYL	DFPPIHF IS	413-436
LRR13	GV	-KLNLKNN	LIKAI	SLRQLKFWP IK	438-462

Figure 3 Alignment of leucine-rich repeats (LRRs) in PmToll. LRRs of PmToll are aligned with the 24-residue prevailing LRR consensus sequence of TLRs (Bell JK *et al.*, 2003). X refers to any amino acid, ϕ is any hydrophobic residue, and L and F are frequently replaced by other hydrophobic residues. Residues that are conserved to the consensus sequence are shaded in grey.

Recently, the function of LvToll has been studied by using RNAi silencing approach and then WSSV or *V. harveyi* challenging. For WSSV challenging, there was no difference in mortality rates between control shrimp and LvToll-silenced shrimp when these two groups were challenged with WSSV. However, when LvToll-silenced shrimp were challenged by *V. harveyi*, there was a significant increase in mortality and bacterial CFU counts. It was concluded that LvToll is an important factor in the shrimp innate immune response to acute *V. harveyi* infection, but not to WSSV (Han-Ching Wang K *et al.*, 2010). In this study, the specific region of the PmToll gene was used to design and construct the stem-loop of a dsRNA-PmToll expression cassette for expression of the dsRNA-PmToll, which is injected into shrimp to withhold the PmToll expression. The results showed that the PmToll receptor gene could be withheld within 24-72 h after dsRNA injection (data not shown). Further experimentation is necessary to establish whether dsRNA can be entered into shrimp after the withholding of the Toll receptor and to study the expression level of immune genes after withholding of the Toll receptor gene, following pathogen infection.

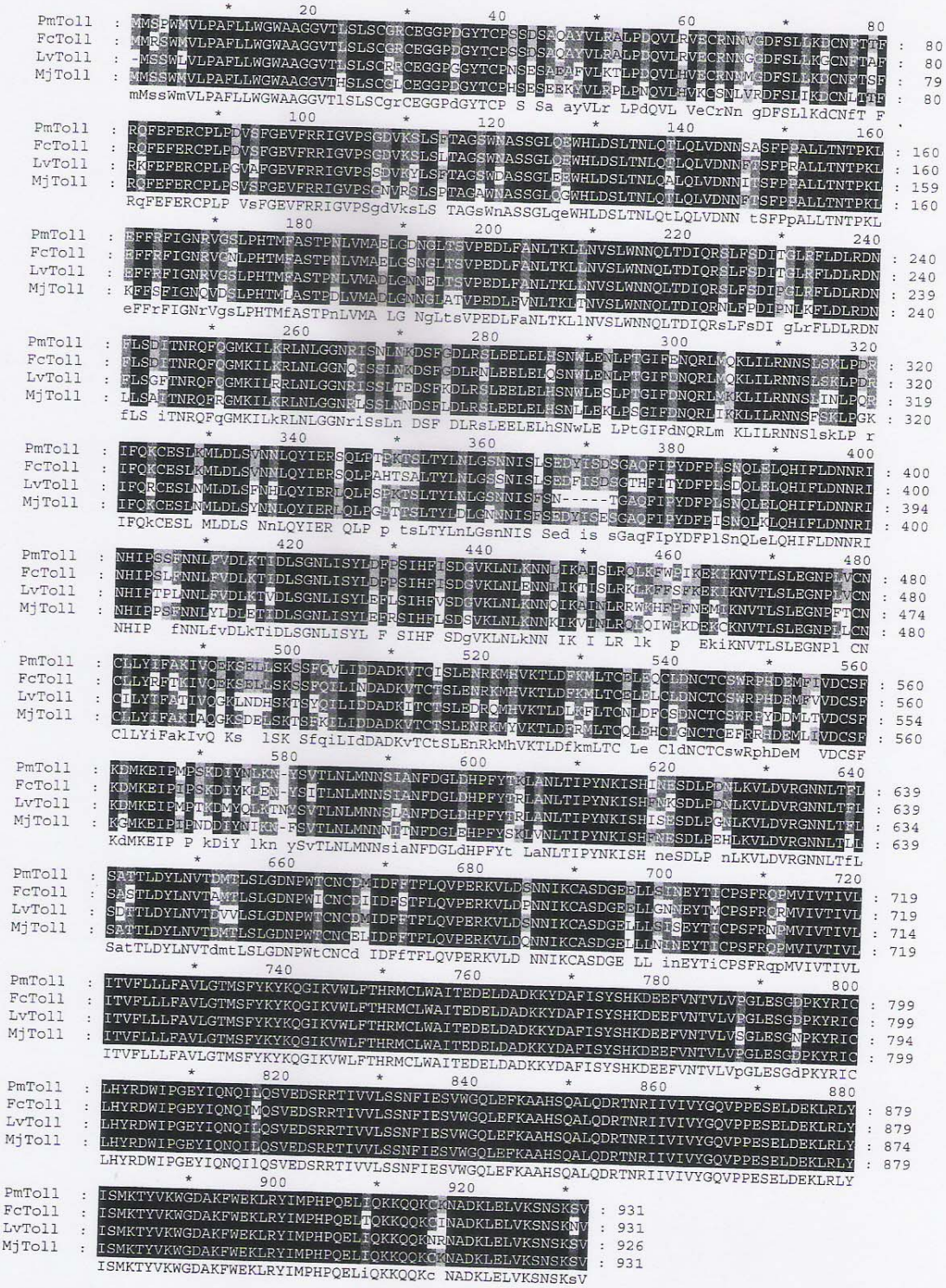

Figure 4 Amino acid sequence alignment of the PmToll, FcToll, LvToll and MjToll. Identical conserved residues are shaded in grey and black

Acknowledgement:
This work was supported by the Thailand Research Fund and Commission for Higher Education. W.A. was supported by grant No. MRG51800160. S.P. is a TRF Senior Research Scholar. We would like to extend our gratitude toward Prof. Anchalee Tassanakajon for suggestions, and also to Mr.Wanlop Chinnirunvong at Institute of Molecular Biosciences for technical assistance.

References:

Akira S, Takeda K and Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2001 ; 2 : 675-680.

Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. *Cell.* 2006 ; 124 : 783-801.

Arts JA, Cornelissen FH, Cijsouw T, Hermsen T, Savelkoul HF, Stet RJ. Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon. Fish Shellfish Immunol.* 2007 ; 23 (3) : 504-513.

Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM.Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* 2003 ; 24 (10) : 528-533.

Bowie A and O'Neill LAJ. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J. Leukoc. Biol.* 2000 ; 67 : 508-514.

Han-Ching Wang K, Tseng CW, Lin HY, Chen IT, Chen YH, Chen YM, Chen TY, Yang HL. RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi. Dev Comp Immunol.* 2010 ; 34 (1) : 49-58.

Hoffmann JA, Kafatos FC, Janeway CA and Ezekowitz RAB. Phylogenetic perspectives in innate immunity. *Science.* 1999 ; 284 : 1313-1318.

Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology.* 2006 ; 211 (4) : 213-236.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM and Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 1996 ; 86 : 973-983.

Loker ES, Adema CM, Zhang SM, Kepler TB. Invertebrate immune systems--not homogeneous, not simple, not well understood. *Immunol Rev.* 2004 ; 198 : 10-24.

Medzhitov R, Preston Hurlburt P and Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997 ; 388 : 394-397.

Mekata T, Kono T, Yoshida T, Sakai M, Itami T. Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus. Fish Shellfish Immunol.* 2008 ; 24 (1) : 122-133.

Ongvarrasopone C, Roshorm Y, Panyim S. A simple and cost effective method to generate dsRNA for RNAi studies in invertebrates. *ScienceAsia.* 2007 ; 33 (1) : 35-39.

Soderhall K and Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity, *Curr. Opin. Immunol.* 1998 ; 10 : 23-28.

Wang PH, Gu ZH, Huang XD, Liu BD, Deng XX, Ai HS, Wang J, Yin ZX, Weng SP, Yu XQ, He JG. An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes. *Mol Immunol.* 2009 ; 46 (8-9) : 1897-1904.

Yang C, Zhang J, Li F, Ma H, Zhang Q, Jose Priya TA, Zhang X, Xiang J. A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol.* 2008 ; 24 (5) : 564-574.

Yang LS, Yin ZX, Liao JX, Huang XD, Guo CJ, Weng SP, Chan SM, Yu XQ, He JG. A Toll receptor in shrimp. *Mol Immunol.* 2007 ; 44 (8) : 1999-2008.

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, Panyim S. YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochem Biophys Res Commun.* 2006 ; 341(2) : 351-356.